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Request for grant of a Patent

Form 1/77 **Patents Act 1977**

❶ Title of invention

1 Please give the title of the invention: Vaccine

❷ Applicant's details

❑ **First or only applicant**

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Agent's name **KATHRYN L. PRIVETT**

Agent's address SmithKline Beecham
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Agent's ADP number 580097400 4

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5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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❼

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8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 [–]

Claim(s) 3 Description 28

Abstract [ ] Drawing(s)

8b Which of the following documents also accompanies the application?

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Patents Form 7/77 - Statement of Inventorship and Right to Grant [–]

Patents Form 9/77 - Preliminary Examination Report [–]

Patents Form 10/77 - Request for Substantive Examination [–]

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VACCINE

FIELD OF INVENTION

The present invention relates to the field of *Streptococcus pneumoniae* capsular polysaccharide antigen vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to.the field of pneumococcal capsular polysaccharide conjugate vaccines, and novel adjuvanted compositions thereof.

BACKGROUND OF INVENTION

Streptococcus pneumoniae is a Gram-positive bacteria that is pathogenic for humans, causing invasive diseases such as pneumonia, bacteremia and meningitis, and diseases associated with colonisation, such as acute Otitis media. It is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are 90 known serotypes of pneumococci, and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. Polysaccharides are T-independent antigens, and can not be processed or presented on MHC molecules to interact with T-cells. They can however, stimulate the immune system through an alternate mechanism which involves cross-linking of surface receptors on B cells.

It was shown in several experiments that protection against invasive pneumococci disease is correlated most strongly with antibody specific for the capsule, and the protection is serotype specific.

Polysaccharide antigen based vaccines are well known in the art. Four that have been licensed for human use include the Vi polysaccharide of *Salmonella typhi*, the PRP polysaccharide from *Haemophilus influenzae*, the tetravalent meningococcal vaccine composed of serotypes A, C, W135 and Y, and the 23-Valent pneumococcal vaccine composed of the polysaccharides corresponding to serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33 (accounting for at least 90% of pneumococcal blood isolates).

The latter three vaccines confer protection against bacteria causing respiratory infections resulting in severe morbidity and mortality in infants, yet these vaccines have not been licensed for use in children less than two years of age because they are inadequately immunogenic in this age group [Peltola *et al.*(1984), N. Engl. J. Med. 310:1561-1566]. *Streptococcus pneumoniae* is the most common cause of invasive bacterial disease and otitis media in infants and young children. Likewise, aged adults mount poor responses to pneumococcal vaccines [Roghmann *et al.*, (1987), J. Gerontol. 42:265-270], hence the increased incidence of bacterial pneumonia in this population [Verghese and Berk, (1983) Medicine (Baltimore) 62:271-285].

Strategies, which have been designed to overcome this lack of immunogenicity in infants, include the linking of the polysaccharide to large immunogenic proteins, which provide bystander T-cell help and which induce immunological memory against the polysaccharide antigen to which it is conjugated. Examples of these proteins which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria and Tetanus toxoids (DT, CRM197 [a mutant of DT], and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD). Pneumococcal glycoprotein conjugate vaccines are currently being evaluated for safety, immunogenicity and efficacy in various age groups.

In addition, it is generally accepted that the protective efficacy of the commercialised pneumococcal vaccine is more or less related to the concentration of antibody induced upon vaccination; indeed, the 23 polysaccharides were accepted for licensure solely upon the immunogenicity of each component polysaccharide (Ed. Williams *et al.* New York Academy of Sciences 1995 pp. 241-249). Therefore further enhancement of antibody responses to the pneumococcal polysaccharides could increase the percentage of infants and elderly responding with protective levels of antibody to the first injection of polysaccharide or polysaccharide conjugate and could reduce the dosage and the number of injections required to induce protective immunity to infections caused by *Streptococcus pneumoniae*.

Since the early 20th century, researchers have experimented with a huge number of compounds which can be added to antigens to improve their immunogenicity in vaccine compositions [reviewed in M.F. Powell & M.J. Newman, Plenum Press, NY, "Vaccine Design – the Subunit and Adjuvant Approach" (1995) Chapter 7 "A Compendium of Vaccine Adjuvants and Excipients"]. Many are very efficient, but cause significant local and systemic adverse reactions that preclude their use in human vaccine compositions. Aluminium-based adjuvants (such as alum, aluminium hydroxide or aluminium phosphate), first described in 1926, remain the only immunologic adjuvants used in human vaccines licensed in the United States.

Aluminium-based adjuvants are examples of the carrier class of adjuvant which works through the "depot effect" it induces. Antigen is adsorbed onto its surface and when the composition is injected the adjuvant and antigen do not immediately dissipate in the blood stream – instead the composition persists in the local environment of the injection and a more pronounced immune response results. Such carrier adjuvants have the additional known advantage of being suitable for stabilising antigens that are prone to breakdown, for instance some polysaccharide antigens.

3D-MPL is an example of a non-carrier adjuvant. Its full name is 3-O-deacylated monophosphoryl lipid A (or 3 De-O-acylated monophosphoryl lipid A or 3-*O*-desacyl-4' monophosphoryl lipid A) and is referred to as 3D-MPL to indicate that position 3 of the reducing end glucosamine is de-O-acylated. For its preparation, see GB 2220211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. It was originally made in the early 1990's when the method to 3-O-deacylate the 4'-monophosphoryl derivative of lipid A (MPL) led to a molecule with further attenuated toxicity with no change in the immunostimulating activity.

3D-MPL has been used as an adjuvant either on its own or, preferentially, combined with a depot-type carrier adjuvant such as aluminium hydroxide, aluminium phosphate or oil-in-water emulsions. In such compositions antigen and 3D-MPL are contained in the same particulate structures, allowing for more efficient delivery of

antigenic and immunostimulatory signals. Studies have shown that 3D-MPL is able to further enhance the immunogenicity of an alum-adsorbed antigen [Thoelen *et al.* Vaccine (1998) 16:708-14; EP 689454-B1]. Precipitated aluminium-based adjuvants are preferred as they are the only adjuvants that are currently used in licensed human vaccines. Accordingly, vaccines containing 3D-MPL in combination with aluminium-based adjuvants are favoured in the art due to their ease of development and speed of introduction onto the market.

MPL (**non** 3-deacylated) has been evaluated as an adjuvant with several monovalent polysaccharide-conjugate vaccine antigens. Coinjection of MPL in saline enhanced the serum antibody response for four monovalent polysaccharide conjugates: pneumococcal PS 6B-tetanus toxoid, pneumococcal PS 12-diphtheria toxoid, and *S. aureus* type 5 and *S. aureus* type 8 conjugated to *Pseudomonas aeruginosa* exotoxin A [Schneerson *et al.* J. Immunology (1991) 147:2136-2140]. The enhanced responses were taught as being antigen-specific. MPL in an oil-in-water emulsion (a carrier type adjuvant) consistently enhanced the effect of MPL in saline due to the presence of MPL and antigen in the same particulate structure, and was considered to be the adjuvant system of choice for optimal delivery of other polysaccharide conjugate vaccines.

Devi *et al.* [Infect. Immun. (1991) 59:3700-7] evaluated the adjuvant effect of MPL (non 3-deacylated) in saline on the murine antibody response to a TT conjugate of *Cryptococcus neoformans* capsular polysaccharide. When MPL was used concurrently with the conjugate there was only a marginal increase in both the IgM- and IgG-specific response to the PS; however MPL had a much larger effect when administered 2 days after the conjugate. The practicality of using an immunization scheme that requires a delay in the administration of MPL relative to antigen, especially in infants, is questionable. The adjuvant effect of MPL with polysaccharides and polysaccharide-protein conjugates appears to be composition-dependent. Again, the incorporation of MPL in a suitable slow-release delivery systems (for instance using a carrier adjuvant) provides a more durable adjuvant effect and circumvents the problem of timing and delayed administration.

In summary, the state of the art has taught that, for particular polysaccharide or polysaccharide-conjugate antigens, where MPL or 3D-MPL is used as an adjuvant, it is advantageously used in conjuction with a carrier adjuvant (for instance the aluminium-based adjuvants) in order to maximise its immunostimulatory effect.

Surprisingly, the present inventors have found that for certain pneumococcal polysaccharide conjugates, the immunogenicity of the vaccine composition is significantly greater when the antigen is formulated with 3D-MPL alone rather than with 3D-MPL in conjunction with an aluminium-based carrier adjuvant. Furthermore the observed improvement is independent of the concentration of 3D-MPL used, and whether the particular conjugates are in a monovalent composition or whether they are combined to form a polyvalent composition.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides an antigenic composition comprising one or more pneumococcal polysaccharide conjugates adjuvanted with 3D-MPL and substantially devoid of aluminium-based adjuvants, wherein at least one of the pneumococcal polysaccharide conjugates is significantly more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant.

Preferred embodiments provided are antigenic compositions comprising conjugates of one or more of the following pneumococcal capsular polysaccharides: serotype 4, 6B, 18C, 19F, and 23F. In such compositions, each of the polysaccharides are surprisingly more immunogenic in compositions comprising 3D-MPL alone compared with compositions comprising 3D-MPL and an aluminium-based adjuvant.

Thus is one embodiment of the invention there is provided a antigenic composition comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 4, 6B, 18C, 19F or 23F conjugated to an immunogenic protein and 3D-MPL adjuvant, wherein the composition is substantially devoid of aluminium-based adjuvants.

In a second embodiment, the present invention provides a combination antigenic composition substantially devoid of aluminium-based adjuvants and comprising 3D-MPL adjuvant and two or more pneumococcal polysaccharide conjugates chosen from the group consisting of: serotype 4; serotype 6B; serotype 18C; serotype 19F; and serotype 23F.

DESCRIPTION OF THE INVENTION

For the purposes of this invention, the term "pneumococcal polysaccharide conjugates of the invention" describes those conjugates of *Streptococcus pneumoniae* capsular polysaccharides which are more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant (for example, conjugates of serotype 4; serotype 6B; serotype 18C; serotype 19F; or serotype 23F).

For the purposes of this invention, the term "substantially devoid of aluminium-based adjuvants" describes a composition which does not contain sufficient aluminium-based adjuvant (for example aluminium hydroxide, and, particularly, aluminium phosphate) to cause any decrease in the immunogenicity of a pneumococcal polysaccharide conjugate of the invention in comparison to an equivalent composition comprising 3D-MPL with no added aluminium-based adjuvant. Quantitities of aluminium-based adjuvant added per dose should preferably be less than 50 μg, more preferably less than 30 μg, still more preferably less than 10 μg, and most preferably there is no aluminium-based adjuvant added to the antigenic compositions of the invention.

For the purposes of this invention, the determination of whether a pneumococcal polysaccharide conjugate is significantly more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant, this should be established as described in Example 2. As an indication of whether a composition is significantly more immunogenic when comprising 3D-MPL alone, the ratio of GMC IgG concentration (as determined in Example 2) between compositions comprising 3D-

MPL alone versus an equivalent composition comprising 3D-MPL in conjunction with aluminium phosphate adjuvant should be more than 5, preferably more than 6, more preferably more than 7, still more preferably more than 9, and most preferably more than 14.

Amongst the problems associated with the polysaccharide approach to vaccination, is the fact that polysaccharides *per se* are poor immunogens. Strategies, which have been designed to overcome this lack of immunogenicity, include the linking (conjugating) of the polysaccharide to large protein carriers, which provide bystander T-cell help. It is preferred that the pneumococcal polysaccharides of the invention are linked to a protein carrier which provides bystander T –cell help. Examples of these immunogenic protein carriers which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria, Diphtheria mutant, and Tetanus toxoids (DT, CRM197 and TT respectively), Keyhole Limpet Haemocyanin (KLH), the purified protein derivative of Tuberculin (PPD), OMPC of *Neisseria meningitidis*, pneumolysin of *Streptococcus pneumoniae*, and protein D of *Haemophilus influenzae* (EP 0 594 610-B).

The present invention in a preferred embodiment provides a protein D from *Haemophilus influenzae*, or fragments thereof, as an immunogenic protein carrier for the pneumococcal polysaccharides of the invention. Fragments suitable for use include fragments encompassing T-helper epitopes. In particular protein D fragments will preferably contain the N-terminal 1/3 of the protein.

In one embodiment the antigenic composition of the invention comprises pneumococcal polysaccharide serotype (PS) 4 conjugated to an immunogenic protein and formulated with 3D-MPL adjuvant, where the composition is substantially devoid of aluminium-based adjuvant. In further embodiments, the antigenic composition comprises PS 6B, 18C, 19F, or 23F, respectively, conjugated to an immunogenic protein and formulated with 3D-MPL adjuvant, where the composition is substantially devoid of aluminium-based adjuvant.

In a still further embodiment of the invention, a combination antigenic composition is provided comprising two or more pneumococcal polysaccharide

conjugates from the group PS 4, PS 6B, PS 18C, PS19F, and PS 23F formulated with 3D-MPL adjuvant, where the composition is substantially devoid of aluminium-based adjuvant.

The immunogenicity of pneumococcal polysaccharide conjugates of the invention is not significantly effected by combination with other pneumococcal polysaccharide conjugates (Example 3). Accordingly, a preferred aspect of the invention provides a combination antigenic composition comprising one or more pneumococcal polysaccharide conjugates of the invention in combination with one or more further pneumococcal polysaccharide conjugates, where the composition is formulated with 3D-MPL adjuvant, but is substantially devoid of aluminium-based adjuvant.

In further preferred embodiments of the invention, combination antigenic compositions are provided which contain at least one and preferably 2, 3, 4 or all 5 of the PS 4, 6B, 18C, 19F, or 23F pneumococcal polysaccharide conjugates, and in addition any combination of other pneumococcal polysaccharide conjugates, which are formulated with 3D-MPL adjuvant but substantially devoid of aluminium-based adjuvant.

Typically the *Streptococcus pneumoniae* combination antigenic composition of the present invention will comprise polysaccharide conjugate antigens, wherein the polysaccharides are derived from at least four serotypes. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 7 serotypes are included in the composition, for example those derived from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. More preferably still, at least 11 serotypes are included in the composition, for example the composition in one embodiment includes the capsular polysaccharide conjugates wherein the polysaccharides are derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. In a preferred embodiment of the invention at least 13 polysaccharide conjugates are included, although more valents, for example 23 valents (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F) are contemplated by the invention.

For elderly vaccination (for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F to the 11 valent antigenic composition above, whereas for infants serotypes 6A and 19A are advantageously included.

The antigenic compositions of the invention are preferably used as vaccine compositions to prevent (or treat) pneumococcal infections.

Further embodiments of the present invention include: the provision of the above antigenic compositions for use in medicine; a method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide conjugate, comprising the steps of administering a safe and effective amount of one of the above antigenic compositions to a patient; and the use of one of the above antigenic compositions in the manufacture of a medicament for the prevention (or treatment) of pneumococcal disease.

For the prevention/amelioration of pneumonia in the elderly (+55 years) population and Otitis media in Infants, (typically 18 months to 5 years), it is a further preferred embodiment of the invention to combine a multivalent *Streptococcus pneumonia* polysaccharide conjugate formulated as herein described with a *Streptococcus pneumoniae* protein or immunologically functional equivalent thereof. Preferred proteins to be included in such a combination vaccine, include but are not limited to: pneumolysin [Mitchell *et al.* Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2.", Mitchell *et al.* Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties.", WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al), WO 99/03884 (NAVA)]; PspA and transmembrane deletion variants thereof (US 5804193 - Briles *et al.*); PspC (WO 97/09994 - Briles et al); PsaA (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*"); Pneumococcal choline binding protein (WO 97/41151); Glyceraldehyde–3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544); HSP 70 (WO 96/40928); M like

protein, SB patent application No. EP 0837130; and adhesin 18627, SB Patent application No. 0834568

The proteins used are preferably selected from the group pneumolysin, PsaA, PspA, CbpA (WO 97/41151) or a combination of two or more such proteins. The present invention also encompasses immunologically functional equivalents to such proteins, e.g. fragments, deletions such as transmembrane deletion variants thereof, fusions, chemically or genetically detoxified derivatives and the like, which are capable of raising substantially the same immune response as the native protein.

The antigenic compositions of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said composition via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts.

Preferably the antigenic compositions (and vaccines) hereinbefore described are lyophilised up until they are about to be used, at which point they are extemporaneously reconstituted with diluent. More preferably they are lyophilsed in the presence of 3D-MPL, and are extemporaneously reconstituted with saline solution.

The amount of conjugate antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 μg of polysaccharide, preferably 0.1-50 μg, more preferably 0.1-10 μg, of which 1 to 5 μg is the most preferable range. For any proteins present in the vaccine, the protein content will typically be in the range 1-100 μg, preferably 5-50μg, most typically in the range 10 - 25μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

EXAMPLES

The examples illustrate, but do not limit the invention.

Example 1

S.pneumoniae capsular polysaccharide:

The 11-valent candidate vaccine includes the capsular polysaccharides serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F which were made essentially as described in EP 72513. Each polysaccharide is activated and derivatised using CDAP chemistry (WO 95/08348) and conjugated to the protein carrier. All the polysaccharides are conjugated in their native form, except for the serotype 3.Its size was reduced by micro-fluidisation.

Protein carrier:

The protein carrier selected is the recombinant protein D (PD) from Non typeable *Haemophilus influenzae*, expressed in *E. coli.*

EXPRESSION OF PROTEIN D

***Haemophilus influenzae* protein D**

Genetic construction for protein D expression

Starting materials

The Protein D encoding DNA

Protein D is highly conserved among *H. influenzae* of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) Infect. Immun. 59: 119-125.

The expression vector pMG1

The expression vector pMG1 is a derivative of pBR322 (Gross *et al.*, 1985) in which bacteriophage λ derived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman *et al.*, 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

The E. coli strain AR58

The *E. coli* strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil$^-$ cI857 ΔH1). N99 and SA500 are *E. coli* K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

The expression vector pMG 1

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilises signals from lambdaphage DNA to drive the transcription and translation of inserted foreign genes. The vector contains the promoter PL, operator OL and two utilisation sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross *et al.*, 1985). Vectors containing the PL promoter, are introduced into an *E. coli* lysogenic host to stabilise the plasmid DNA. Lysogenic host strains contain replication-defective lambdaphage DNA integrated into the genome (Shatzman *et al.*, 1983). The chromosomal lambdaphage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981).

The E. coli strain AR58

The AR58 lysogenic *E. coli* strain used for the production of the protein D carrier is a derivative of the standard NIH *E. coli* K12 strain N99 (F^- su^- galK2, $lacZ^-$ thr^-). It contains a defective lysogenic lambdaphage (galE::TN10, lambdaKil$^-$ cI857 ΔH1). The Kil$^-$ phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the lambdaphage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil$^-$ cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin resistance in the adjacent galE gene.

Construction of vector pMGMDPPrD

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNSI) was used to construct pMGMDPPrD. The protein D gene was amplified by PCR from the pHIC348 vector (Janson *et al.* 1991) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino acids of the NS1 protein followed by the PD protein. This vector was labeled pMGNS1PrD.

Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

-----MDP SSHSSNMANT-----

NS1 Protein D

The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by heat shock at 37 °C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant *E. coli* strain is referred to as ECD4.

Expression of protein D is under the control of the lambda P_L promoter/ O_L Operator. The host strain AR58 contains a temperature-sensitive cI gene in the genome which blocks expression from lambda P_L at low temperature by binding to O_L. Once the temperature is elevated cI is released from O_L and protein D is expressed. At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D was performed as follows. The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final $OD_{650} = 60$. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate is clarified by centrifugation and cell debris are removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 μm membrane.

Chemistry:

Activation and coupling chemistry:

The activation and coupling conditions are specific for each polysaccharide. These are given in Table 1. Native polysaccharide (except for PS3) was dissolved in NaCl 2M or in water for injection. The optimal polysaccharide concentration was evaluated for all the serotypes.

From a 100 mg/ml stock solution in acetonitrile, CDAP (CDAP/PS ratio 0.75 mg/mg PS) was added to the polysaccharide solution. 1.5 minute later, 0.2M triethylamine was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 2 minutes at 25 °C. Protein D (the quantity depends on the initial PS/PD ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for 1 hour. The reaction was then quenched with glycine for 30 minutes at 25 °C and overnight at 4 °C.

The conjugates were purified by gel filtration using a Sephacryl 500HR gel filtration column equilibrated with 0.2M NaCl.

The carbohydrate and protein content of the eluted fractions was determined. The conjugates were pooled and sterile filtered on a 0.22μm sterilizing membrane. The PS/Protein ratios in the conjugate preparations were determined.

Characterisation:

Each conjugate was characterised and met the specifications described in Table 2. The polysaccharide content (μg/ml) was measured by the Resorcinol test and the protein content (μg/ml) by the Lowry test. The final PS/PD ratio (w/w) is determined by the ratio of the concentrations.

Residual DMAP content (ng/μg PS):

The activation of the polysaccharide with CDAP introduces a cyanate group in the polysaccharide and DMAP (4-dimethylamino-pyridin) is liberated. The residual DMAP content was determined by a specific assay developed at SB.

Free polysaccharide content (%):

The free polysaccharide content of conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with α-PD antibodies and saturated ammonium sulfate, followed by a centrifugation.

An α-PS/α-PS ELISA was used for the quantification of free polysaccharide in the supernatant . The absence of conjugate was also controlled by an α-PD/α-PS ELISA.

Example 2 – Study of the Effect of Advanced Adjuvants on the Immunogenicity of the 11-Valent Pneumococcal PS-PD Conjugate Vaccine in Infant Rats

Infant rats were immunised with 11 valent pneumococcal PS-PD conjugate vaccine at a dosage of 0.1 μg each polysaccharide (made according to the method of Example 1), and using the following adjuvant formulations: none, $AlPO_4$, 3D-MPL, 3D-MPL on $AlPO_4$.

The formulation with only 3D-MPL was statistically (and surprisingly) more immunogenic (greatest GMC IgG) than for the other formulations for 5 out of 11 antigens. This was true both at high and low concentrations of 3D-MPL.

Opsonophagocytosis confirmed the GMC results.

Materials and Methods

Immunisation Protocol

Infant OFA rats were randomised to different mothers and were 7 days old when they received the first immunisation. They received 2 additional immunisations 14 and 28 days later. A bleed was performed on day 56 (28 days post III). All vaccines were injected s.c., and there were 10 rats per vaccine group.

The rats were immunised with an 11 valent pneumococcal conjugate vaccine comprising the following polysaccharide serotypes conjugated onto protein D: 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F.

Formulation

To examine the effect of different advanced adjuvants, the dosage of conjugate was held constant at 0.1 μg of each polysaccharide, and the adjuvants $AlPO_4$ and 3D-MPL were formulated in different dosages and combinations, including no adjuvant at all. These are listed numerically in Table 3 for reference.

Adsorption on AlPO4

The concentrated, adsorbed monovalents were prepared according to the following procedure. 50 μg $AlPO_4$ (pH 5.1) was mixed with 5 μg conjugated polysaccharides for 2 hours. The pH was adjusted to pH 5.1 and the mixture was left for a further 16 hours. 1500mM NaCl was added to make up the salt concentration to 150 mM. After 5 minutes 5 mg/mL 2-phenoxyethanol was added. After a further 30 minutes the pH was adjusted to 6.1, and left for more than 3 days at 4 °C.

Preparation of diluents

Three diluents were prepared in NaCl 150 mM/ 5 mg/mL phenoxyethanol

A: $AlPO_4$ at 1 mg/ml.

B: 3D-MPL on $AlPO_4$ at 250 and 1000 μg/ml respectively Weight ratio 3D-MPL/$AlPO_4$ = 5/20

C: 3D-MPL on $AlPO_4$ at 561 and 1000μg/ml respectively Weight ratio 3D-MPL/$AlPO_4$ = 50/89

Preparation of adsorbed undecavalent

The eleven concentrated, adsorbed PS-PD monovalents were mixed at the correct ratio. The complement of $AlPO_4$ was added as the diluent A. When required, 3D-MPL was added either as an aqueous solution (non adsorbed, Way 1 see below) or as the diluent B or C (3D-MPL adsorbed on $AlPO_4$ at 2 doses, Way 2, see below).

Way 1

3D-MPL was added to the combined adsorbed conjugates as an aqueous suspension. It was mixed to the undecavalent for 10 minutes at room temperature and stored at 4 °C until administration.

Way 2

3D-MPL was preadsorbed onto $AlPO_4$ before addition to the combined adsorbed conjugates (diluent B and C). To prepare 1 ml of diluent, an aqueous suspension of 3D-MPL (250 or 561 µg) was mixed with 1 mg of $AlPO_4$ in NaCl 150 mM pH 6.3 for 5 min at room temperature. This solution was diluted in NaCl pH 6.1/phenoxy and incubated overnight at 4 °C.

Preparation of non-adsorbed undecavalent

The eleven PS-PD conjugates were mixed and diluted at the right ratio in NaCl 150 mM pH 6.1, phenoxy. When required, 3D-MPL was added as a solution (non adsorbed).

The formulations for all injections were prepared 18 days before the first administration.

ELISA

The ELISA was performed to measure rat IgG using the protocol derived from the WHO Workshop on the ELISA procedure for the quantitation of IgG antibody against *Streptococcus pneumoniae* capsular polysaccharides in human serum. In essence, purified capsular polysaccharide is coated directly on the microtitre plate. Serum samples are pre-incubated with the cell-wall polysaccharide common to all pneumococcus (substance C) and which is present in ca. 0.5% in pneumococcal polysaccharides purified according to disclosure (EP 72513 B1). Jackson ImmunoLaboratories Inc. reagents were employed to detect bound murine IgG. The titration curves were referenced to internal standards (monoclonal antibodies) modeled by logistic log equation. The calculations were performed using SoftMax Pro software. The maximum absolute error on these results expected to be within a factor of 2. The relative error is less than 30%.

Opsonophagocytosis

Opsonic titres were determined for serotypes 3, 6B, 7F, 14, 19F and 23F using the CDC protocol (*Streptococcus pneumoniae* Opsonophagocytosis using Differentiated HL60 cells, version 1.1) with purified human PMN and baby rabbit complement. Modification included the use of in-house pneumococcal strains, and the phagocytic HL60 cells were replaced by purified human PMN. In addition, 3 mm glass beads were added to the microtitre wells to increase mixing, and this allowed reduction of the phagocyte:bacteria ratio which was recommended to be 400.

Results

IgG Concentrations

The geometric mean IgG concentrations determined for every serotype, and PD are shown in Tables 4 to 10. For serotypes 6B, 14, 19F and 23F, previous results obtained using a tetravalent formulation are included for comparison.

The highest IgG concentrations have been highlighted in Tables 4 to 10. The statistical p value for 3D-MPL compositions vs. 3D-MPL/ $AlPO_4$ compositions is in Table 11. Adjuvant formulation number 4 (non-adsorbed conjugates with high dose 3D-MPL) that gives the highest GMC's for 9 out of 11 cases. In 5/11 cases, MPL at the low dose is the second most immunogenic. In addition, adjuvantation gives higher GMC's than by modifying the dose for all serotypes (data not shown), and this is statistically significant for serotypes 4, 6B, 7F, 18C and 23F ($p < 0.05$ from 95% CI).

Opsonophagocytosis

Opsonophagocytosis results on pooled sera is shown for serotypes 3, 6B, 7F, 14, 19F and 23F in Tables 4 to 8. For the most part, these opsonic titres confirm the GMC IgG. Indeed, the correlation with IgG concentration is greater than 85% for serotypes 6B, 19F, 23F (data not shown). For serotype 3, it is important to note that only the 3D-MPL group induced opsonic activity above the threshold.

Conclusions

In this experiment, it was unexpected that the use of 3D-MPL alone would induce the highest IgG concentrations.

The maximal GMC IgG obtained with modifying the adjuvant was compared with the maximal GMC obtained by modifying the PS dosage, and it was found that 3D-MPL could induce significantly higher responses in 5/11 serotypes.

Table 11 shows that when 3D-MPL and 3D-MPL/ $AlPO_4$ compositions are compared (comparing the process of formulation, and the dose of 3D-MPL), 5 of the polysaccharide conjugates are significantly improved, in terms of immunogenicity, when formulated with just 3D-MPL rather than 3D-MPL plus $AlPO_4$: PS 4, PS 6B, PS 18C, PS 19F, and PS 23F.

Example 3 – Study of the effect of combination on the immunogenicity of PS 4, PS 6B, PS 18C, PS 19F, and PS 23F conjugates in adult rats

Adult rats were immunised with pneumococcal polysaccharide-protein D conjugate vaccines either individually, or combined in a multivalent composition (either tetra-, penta-, hepta-, or decavalent). Groups of 10 rats were immunised twice 28 days apart, and test bleeds were obtained on day 28 and day 42 (14 days after the 2nd dose).

The sera were tested by ELISA for IgG antibodies to the pneumococcal polysaccharides. All conjugates induced specific IgG antibodies as measured by ELISA. Table 12 shows the effect of combination of monovalent PS 6B, PS 18C, PS 19F, and PS 23F protein D conjugates on their immunogenicity in adult rats, as measured by IgG concnetration at 14 days post 2nd dose.

Statistical analysis was performed on all samples to determine if differences in antibody concentration upon combination were significant. The combination of any of serotypes PS 6B, PS 18C, PS 19F, and PS 23F protein D conjugates in a multivalent vaccine did not significantly change their immunogenicity.

Table 1
Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D conjugates

Serotype	1	3 (µfluid.)	4	5	6B	7F
PS conc.(mg/ml)	2.0	3.0	2.0	7.5	5.4	3.0
PS dissolution	NaCl 2M	NaCl 2M	H_2O	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/1	1/1	1/1	1/1	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$pH_a=pH_c=pH_q$	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0

Serotype	9V	14	18C	19F	23F
PS conc.(mg/ml)	2.5	2.5	2.0	4.0	3.3
PS dissolution	NaCl 2M	NaCl 2M	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.75	1/0.75	1/1	1/0.5	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75
$pH_a=pH_c=pH_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	10/9.5/9.0	9.0/9.0/9.0

TABLE 2

Criteria	D01PDJ227	D03PDJ236	D4PDJ228	D5PDJ235	D6PDJ209
Ratio PS/Prot (w/w)	1/0.66	1/1.09	1/0.86	1/0.86	1/0.69
Free polysac. content (%) <10 %	1	1	7	9	0
Free protein content (%) <15 %	8	<1	19	21	9
DMAP content (ng/µg PS) < 0.5 ng/µg PS	0.2	0.6	0.4	1.2	0.3
Molecular size (K_{av})	0.18	0.13	0.12	0.11	0.13
Stability	no shift	no shift	no shift	low shift	no shift

	D07PDJ225	D09PDJ222	D14PDJ202	D18PDJ221	D19PDJ206	D23PDJ212
Ratio PS/Prot (w/w)	1/0.58	1/0.80	1/0.68	1/0.62	1/0.45	1/0.74
Free polysac. content (%) <10 %	1	<1	<1	4	4	0
Free protein content (%) <15 %	8	0.3	3	21	10	12
DMAP content (ng/µg PS) <0.5 ng/µg PS	0.1	0.6	0.3	0.2	0.1	0.9
Molecular size (K_{av})	0.14	0.14	0.17	0.10	0.12	0.12
Stability	no shift	no shift	no shift	no shift	shift	no shift

Table 3. Summary Table of Adjuvant Formulations tested with 11-Valent Pneumococcal PS-PD in Infant Rats

Group	AlPO4	MPL	Method	Description
1				None
2	100			AlPO4
3		5		MPL low
4		50		MPL High
5	100	5	Way 1	Way 1 low
6	100	50	Way 1	Way 1 high
7	100	5	Way 2	Way 2 low
8	100	50	Way 2	Way 2 high

5

Table 4. Serotype 6B Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AlPO4 µg	MPL µg	Method	6B GMC IgG (µg/ml)	6B Sero-conversion	6B Opso Titre*	6B GMC IgG (µg/ml)	6B Sero-conversion	6B Opso Titre*
				Tetravalent			Undecavalent		
1				0.047	2/10	12.5	0.004	1/10	<6.25
2	100			0.048	4/10	65	0.019	4/10	<6.25
3		5					1.345	10/10	43
4		**50**					**4.927**	**10/10**	**192**
5	100	5	1				0.042	7/10	<6.25
6	100	50	1				0.255	10/10	<6.25
7	100	5	2	0.033	3/10	<6.25	0.048	8/10	<6.25
8	100	50	2				0.057	8/10	<6.25

10

Table 5. Serotype 14 Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AIPO4	MPL	Method	14 GMC IgG (µg/ml)	14 Sero-con-version	14 Opsonic Titre*	14 GMC IgG (µg/ml)	14 Sero-con-version	14 Opsonic Titre*
				Tetravalent			Undecavalent		
1				0.046	3/10	64	0.022	3/10	<6.25
2	100			0.99	10/10	88	0.237	8/10	27
3		5					0.233	10/10	41
4		50					0.676	10/10	81
5	100	5	1				0.460	9/10	67
6	100	50	1				0.477	10/10	98
7	100	5	2	0.81	10/10	49	0.165	8/10	81
8	**100**	**50**	**2**				**1.611**	**10/10**	**133**

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Table 6. Serotype 19F Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AIPO4 µg	MPL µg	Method	19F GMC IgG (µg/ml)	19F Sero-con-version	19F Opsonic Titre*	19F GMC IgG (µg/ml)	19F Sero-con-version	19F Opsonic Titre*
				Tetravalent			Undecavalent		
1				0.04	2/10	64	0.021	2/10	<6.25
2	100			1.07	9/10	367	0.222	7/10	79
3		5					4.028	10/10	296
4		**50**					**21.411**	**10/10**	**1276**
5	100	5	1				1.649	10/10	172
6	100	50	1				2.818	10/10	208
7	100	5	2	1.09	10/10	193	0.766	10/10	323
8	100	50	2				3.539	10/10	241

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Table 7. Serotype 23F Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AlPO4 µg	MPL µg	Method	23F GMC IgG (µg/ml)	23F Sero-conversion	23F Opsonic Titre*	23F GMC IgG (µg/ml)	23F Sero-conversion	23F Opsonic Titre*
				Tetravalent			Undecavalent		
1				0.06	2/10	<6.25	0.152	3/10	<6.25
2	100			0.29	10/10	70	0.56	8/10	<6.25
3		**5**					**2.296**	**9/10**	**389**
4		**50**					**4.969**	**10/10**	**>1600**
5	100	5	1				0.462	5/10	17
6	100	50	1				0.635	8/10	54
7	100	5	2	0.38	10/10	<6.25	0.203	3/10	18
8	100	50	2				0.501	7/10	43

Table 8. Serotypes 3 and 7F Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants

Group	AlPO4 µg	MPL µg	Method	3 GMC IgG (µg/ml)	3 Sero-conversion	3 Opsonic Titre*	7F GMC IgG (µg/ml)	7F Sero-conversion	7F Opsonic Titre*
1				0.003	1/10	<6.25	0.040	7/10	<6.25
2	100			0.008	6/10	<6.25	0.25	9/10	43
3		**5**		**0.070**	**10/10**	**<6.25**	**2.435**	**10/10**	**477**
4		**50**		**0.108**	**10/10**	**18**	**2.569**	**10/10**	**332**
5	100	5	1	0.015	10/10	<6.25	0.579	10/10	54
6	100	50	1	0.027	10/10	<6.25	0.611	9/10	59
7	100	5	2	0.006	10/10	<6.25	0.154	8/10	30
8	100	50	2	0.034	10/10	<6.25	0.638	9/10	140

Table 9. Serotypes 1, 4 and 5 Geometric Mean IgG Concentration and Seroconversion on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants

Group	AlPO4 µg	MPL µg	Method	1 GMC IgG (µg/ml)	1 Sero-con-version	4 GMC IgG (µg/ml)	4 Sero-con-version	5 GMC IgG (µg/ml)	5 Sero-con-version
1				0.026	4/10	0.005	0/10	0.040	3/10
2	100			0.282	8/10	0.052	5/10	0.774	9/10
3		5		1.614	10/10	**3.452**	**10/10**	7.927	10/10
4		**50**		2.261	10/10	**7.102**	**10/10**	**13.974**	**10/10**
5	100	5	1	0.568	10/10	0.676	10/10	3.015	10/10
6	100	50	1	1.430	10/10	0.419	9/10	5.755	10/10
7	100	5	2	0.478	10/10	0.267	9/10	2.062	10/10
8	100	50	2	1.458	10/10	0.423	10/10	5.009	10/10

Table 10. Serotypes 9V, 18C and PD Geometric Mean IgG Concentration and Seroconversion on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants

Group	AlPO4 µg	MPL µg	Method	9V GMC IgG (µg/ml)	9V Sero-con-version	18C GMC IgG (µg/ml)	18C Sero-con-version	PD GMC IgG (µg/ml)	PD Sero-con-version
1				0.018	0/10	0.013	1/10	0.003	0/10
2	100			0.489	6/10	0.092	5/10	0.993	10/10
3		5		0.482	7/10	**6.560**	**10/10**	3.349	10/10
4		**50**		**11.421**	**10/10**	**14.023**	10/10	5.446	10/10
5	100	5	1	2.133	9/10	0.690	10/10	**11.407**	**10/10**
6	100	50	1	2.558	10/10	1.771	10/10	1.258	10/10
7	100	5	2	1.536	10/10	0.528	10/10	1.665	8/10
8	100	50	2	2.448	9/10	0.980	10/10	5.665	10/10

Table 11: The statistical significance (p value) of whether certain pneumococcal polysaccharide conjugates had improved immunogenicty when formulated with 3D-MPL alone versus with 3D-MPL/AlPO4. A p value under 0.01 is considered highly significant. Way 1 and Way 2 indicate the method of formulation.

serotype	50 µg 3D-MPL v 3D-MPL/$AlPO_4$		5 µg 3D-MPL vs 3D-MPL/$AlPO_4$	
	Way 1	Way 2	Way 1	Way 2
1	0.3	0.05	0.079	0.11
3	0.075	0.01	0.27	0.008
4	0.002	0.0003	0.02	0.003
5	0.04	0.002	0.1	0.12
6B	0.001	0.0001	0.001	0.0006
7F	0.13	0.15	0.01	0.005
9V	0.02	0.02	0.1	0.04
14	0.65	0.21	0.3	0.66
18C	0.0008	0.0002	0.006	0.004
19F	0.0009	0.006	0.21	0.04
23F	0.002	0.0004	0.01	0.0004

Table 12: Geometric Mean IgG concentration (µg/mL) on day 14 post 2nd dose after immunisation of adult rats with 1.0 µg polysaccharide-protein D conjugate alone or combined in tetravalent, pentavalent, heptavalent or decavalent vaccine. These data are combined from 5 separate experiments.

Serotypes Vaccines	4 H	6B T	18C H	19F T	23F T
Alone	9.3	0.11	15	5.2	2.5
Combined	4	0.23	3.7	3.7	2.8

T: combined in tetravalent (T) (PS 6B, 14, 19F, 23F), pentavalent (T plus PS 3), heptavalent (H) (T plus PS 4, 9V and 18C), and decavalent (H plus PS 1, 5 and 7F) combination vaccines. H: combined in heptavalent (H) (T plus PS 4, 9V and 18C), and decavalent (H plus PS 1, 5 and 7F) combination vaccines.

We claim:

1. An antigenic composition comprising one or more *Streptococcus pneumoniae* capsular polysaccharide conjugates adjuvanted with 3D-MPL and substantially devoid of aluminium-based adjuvants, wherein at least one of the *Streptococcus pneumoniae* polysaccharide conjugates is significantly more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant.

2. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 4 conjugated to an immunogenic protein.

3. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 6B conjugated to an immunogenic protein.

4. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 18C conjugated to an immunogenic protein.

5. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 19F conjugated to an immunogenic protein.

6. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 23F conjugated to an immunogenic protein.

7. A combination antigenic composition substantially devoid of aluminium-based adjuvants and comprising 3D-MPL adjuvant and two or more pneumococcal polysaccharide conjugates chosen from the group consisting of:

    serotype 4;

    serotype 6B;

    serotype 18C;

serotype 19F; and

serotype 23F.

8. The antigenic compositions of claims 1-7 wherein the *Streptococcus pneumoniae* capsular polysaccharides are conjugated to a protein chosen from the group consisting of:

tetanus toxoid;

OMPC of *Neisseria meningitidis*;

diphtheria toxoid;

pneumolysin of *Streptococcus pneumoniae*; or

CRM197.

9. The antigenic compositions of claims 1-7 wherein the *Streptococcus pneumoniae* capsular polysaccharides are conjugated to a protein D of *Haemophilus influenzae*.

10. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 6B, 14, 19F and 23F.

11. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 4, 6B, 9V, 14, 18C, 19F and 23F.

12. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F.

13. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 3, 4, 5, 6B, 7F, 8, 9V, 12F, 14, 18C, 19F and 23F.

14. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F.

15. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

16. A lyophilsed antigenic composition according to claims 1-15.

17. The antigenic composition of claims 1-16, which is a vaccine composition.

18. A method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide conjugate, said method comprising administering a safe and effective amount of an antigenic composition as claimed herein to a patient.

19. Use of an antigenic composition as claimed herein in the manufacture of a medicament for the prevention of pneumococcal disease.